Plant Physiol. (1993) 101: 37–47

Photosynthetic Fractionation of the Stable Isotopes of Oxygen and Carbon[1]

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Isotope discrimination during photosynthetic exchange of O_2 and CO_2 was measured using enzyme, thylakoid, and whole cell preparations. Evolved oxygen from isolated spinach thylakoids was isotopically identical (within analytical error) to its source water. Similar results were obtained with *Anacystis nidulans* Richter and *Phaeodactylum tricornutum* Bohlin cultures purged with helium. For consumptive reactions, discrimination (Δ, where $1 + \Delta/1000$ equals the isotope effect, k^{16}/k^{18} or k^{12}/k^{13}) was determined by analysis of residual substrate (O_2 or CO_2). The Δ for the Mehler reaction, mediated by ferredoxin or methylviologen, was 15.3‰. Oxygen isotope discrimination during oxygenation of ribulose-1,5-bisphosphate (RuBP) catalyzed by RuBP carboxylase/oxygenase (Rubisco) was 21.3‰ and independent of enzyme source, unlike carbon isotope discrimination: 30.3‰ for spinach enzyme and 19.6 to 23‰ for *Rhodospirillum rubrum* and *A. nidulans* enzymes, depending on reaction conditions. The Δ for O_2 consumption catalyzed by glycolate oxidase was 22.7‰. The expected overall Δ for photorespiration is about 21.7‰. Consistent with this, when *Asparagus sprengeri* Regel mesophyll cells approached the compensation point within a sealed vessel, the $\delta^{18}O$ of dissolved O_2 came to a steady-state value of about 21.5‰ relative to the source water. The results provide improved estimates of discrimination factors in several reactions prominent in the global O cycle and indicate that photorespiration plays a significant part in determining the isotopic composition of atmospheric oxygen.

On a global scale, photosynthetic O_2 is added to the atmosphere at a rate equal to its consumption. That is, the O_2 concentration of the earth's atmosphere reflects a global "compensation point." All free O_2 is produced by photosynthetic organisms that, in turn, also consume a large proportion. Associated with the global O_2 compensation point is an isotopic compensation point. The $^{18}O/^{16}O$ of atmospheric O_2 is 1.0235 times that of seawater (i.e. relative to V-SMOW, the $\delta^{18}O$ of air is +23.5‰)[3] (Kroopnick and Craig, 1972). This steady-state difference in isotopic composition between atmospheric O_2 and its ultimate source is termed the "Dole effect" (Dole, 1935).

Previous attempts to explain the Dole effect have emphasized isotope discrimination in photosynthesis and microbial respiration (Dole et al., 1947; Lane and Dole, 1956; Schleser, 1978), and fractionation during transpirational enrichment of leaf water (Förstel, 1978). These treatments are incomplete because there is still controversy concerning discrimination in O_2 production and there are several mechanisms of O_2 uptake by plants. The process of photorespiration, estimated to account for about 30% of gross global O_2 uptake, has the potential for isotope discrimination by glycolate oxidase (EC 1.1.3.1) and during oxygenation of RuBP by Rubisco (EC 4.1.1.39). In addition, the Mehler reaction may account for as much as 10% of total O_2 uptake (Canvin et al., 1980; Furbank et al., 1982), and another 20% of global O_2 consumption is attributable to plant mitochondrial respiration, a portion of which is mediated by an enzyme other than Cyt oxidase (i.e. the "alternative oxidase"). Thus, approximately 60% of global O_2 consumption is mediated by various plant processes, and only the remaining 40% is by nonphotosynthetic organisms, principally microbes.

Despite the fact that plant life plays a central role in the global O cycle, isotope discrimination factors related to O_2 evolution and consumption by plants are poorly defined. This information is essential to an understanding of variations in the natural abundance of ^{18}O and in possible applications to biogeochemical problems, such as in estimating the gross production of marine ecosystems (Bender and Grande, 1987). We have recently reported differential discrimination during dark respiration by plants, mediated by either the Cyt pathway or the alternative pathway (Guy et al., 1989a). Our present focus is on O_2 exchange (i.e. production and consumption) in the light. We first examine discrimination by individual reactions in vitro, and then scale up to their simultaneous occurrence at the whole cell level in microcosm experiments that provide simplified analogs to the global O cycle.

MATERIALS AND METHODS

Isotope Fractionation—General Procedures

For uptake reactions, discrimination against ^{18}O or ^{13}C was measured within a closed reaction vessel by examining

[1] Carnegie Institution of Washington-Department of Plant Biology publication No. 1143. This work was supported by U.S. Department of Energy grant FG0586 ER3563 to J.A.B. and M.L.F.

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[3] Isotope abundances are expressed in per mil (‰) units using the δ notation, $\Delta X = [(R_{sample}/R_{standard}) - 1] \cdot 1000$, where X is ^{18}O or ^{13}C, R_{sample} is the sample $^{18}O/^{16}O$ or $^{13}C/^{12}C$ ratio, and $R_{standard}$ is the $^{18}O/^{16}O$ or $^{13}C/^{12}C$ ratio of the standard. For $\delta^{18}O$, the standard is V-SMOW. For $\delta^{13}C$ the standard is PeeDee belemnite.

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Abbreviations: DIC, total dissolved inorganic carbon; DPC, diphenylcarbazide; *n*, sample size; RuBP, ribulose-1,5-bisphosphate; V-SLAP, Vienna-standard light antarctic precipitation; V-SMOW, Vienna-standard mean ocean water.

changes in the isotopic composition of dissolved substrate, either O_2 or CO_2, as it was consumed. Theoretical considerations, equipment, and procedures have been detailed in our earlier work (Guy et al., 1989a, 1992). Briefly, suspensions of cells, enzymes, or thylakoids were incubated in a closed vessel that could be sampled sequentially two to five times per experiment. Dissolved O_2 concentration was monitored with an O_2 electrode. Samples taken directly into an evacuated bulb mounted at one end of a vacuum line were immediately discharged into a receiving vessel containing 5 mL of H_3PO_4 and 1 g of sulfosalicylic acid to halt biochemical reactions and prevent frothing of denatured proteins. Dissolved gases were then stripped from solution by bubbling with zero-grade helium. After removing water vapor by passage through a dry ice:ethanol trap, CO_2 was collected on a series of nine loops passing in and out of two liquid N_2 baths. O_2 was trapped on Molecular Sieve 5A and purified by chromatography before conversion to CO_2 by reaction with graphite at 750°C. Yields of CO_2 were determined manometrically and used to calculate substrate depletion during the course of O_2 or CO_2 uptake reactions. Isotope analysis was performed on Nuclide 6–60 ratio mass spectrometers with a precision of ±0.1‰.

O_2 Evolution

O_2 produced from water in photosynthesis was trapped and prepared for isotopic analysis as above. Buffers were prepared from water spiked with $H_2{}^{18}O$ such that the isotope ratio was similar to that of air to minimize errors due to inevitable leaks or carry-over of atmospheric O_2 within preparations. Plant materials were prepared with the same batch of water as used in the experiments.

Two separate approaches were used to prevent simultaneous O_2 uptake during O_2 evolution. In the first, O_2 production was carried out in vitro with thylakoid preparations provided with an effective Hill reagent. Thylakoids were isolated from fresh market spinach (Nolan and Smillie, 1976) and injected into degassed 50 mM K-phosphate buffer (pH 7.5) with 20 mM methylamine and 4 mM $K_3Fe(CN)_6$. Samples were removed following illumination for 3 to 12 min, and an aliquot of remaining buffer was kept for isotopic analysis of the source water.

In the second approach, illuminated algal cultures were stripped of O_2 as it was produced by continuously sparging with zero-grade helium. Experiments were with *Anacystis nidulans* Richter (R2; *Synechococcus* sp. strain 6301) in BG11 medium (Stanier et al., 1971) buffered to pH 8 with 20 mM Hepes, and *Phaeodactylum tricornutum* Bohlin in pH 8 artificial sea water (Darley and Volcani, 1969). The initial $NaHCO_3$ concentration was 20 mM. Cultures were vigorously bubbled with helium in a 1-L glass vessel with a fritted glass disk at the bottom. Part of the helium stream then entered the preparation line. O_2 was collected for 20 to 40 min. Waters were sampled before and after each experiment.

Rubisco

Spinach Rubisco was prepared according to Hall and Tolbert (1978). Preparations of *A. nidulans* and *Rhodospirillum rubrum* Rubisco, expressed in *Eschericia coli*, were provided courtesy of George Lorimer and Stephen Gutteridge (DuPont). Carbon (in CO_2) and oxygen (in O_2) isotopes were studied together in some experiments and separately in others. Most experiments were in 50 mM Bicine buffer with 20 to 35 μg/mL carbonic anhydrase. Oxygenation-only experiments were initiated in 2 mM $NaHCO_3$ and 1.2 mM O_2. O_2 was excluded from carboxylation-only experiments, and $NaHCO_3$ was 2 to 5 mM. These bicarbonate concentrations and the absence of ambient air precluded the possibility of significant mass 44 interference due to N_2O contamination. Reactions were controlled by addition of aliquots of a solution of RuBP (240 mM; synthesized according to Horecker et al., 1957).

Most experiments with spinach Rubisco (250 μg/mL purified enzyme) were at pH 8.5 and 20 mM $MgCl_2$. However, one carboxylation experiment was done in Hepes buffer (pH 7.6) with only 5 mM $MgCl_2$. Studies of *R. rubrum* Rubisco (80 μg/mL) were performed at pH 7.9, with carboxylation examined at 2 and 25 mM $MgCl_2$ and oxygenation examined at 20 mM $MgCl_2$. All *A. nidulans* Rubisco experiments were at pH 8.1 and 25 mM $MgCl_2$, with enzyme supplied at 25 μg/mL.

Glycolate Oxidase

O_2 uptake by spinach glycolate oxidase (Sigma) (10–15 μg/mL) was in O_2-saturated 50 mM Tris buffer (pH 8.3) with 70 μM flavin mononucleotide and 2 to 4 mM glycolate. The reaction vessel was kept dark to prevent photooxidation of the flavin mononucleotide. Experiments were done with and without added catalase (10 μg/mL). In the absence of catalase, NaN_3 and KCN were both present at 1 mM.

Mehler Reaction

The uptake of O_2 by reduced Fd or methylviologen was studied using chloroplast membranes devoid of O_2 evolution but able to support normal electron transport in the presence of the alternative electron donor, DPC. Spinach thylakoids were prepared as above, but pellets (from 16 leaves) were resuspended in 20 mL of 1 M Tris buffer (pH 8.0) and left in the dark for 20 min with occasional mixing (Yamashita and Butler, 1968). After centrifugation at 1200*g* for 10 min, the Tris-washed thylakoids were rinsed twice by centrifugation in reaction buffer (5 mM Hepes [pH 7.0], 200 mM sucrose, 2 mM $MgCl_2$). Spinach Fd was extracted according to the procedures of Rao et al. (1971) except that hydroxyapatite chromatography was omitted. Fractions containing Fd eluting from the final DEAE-cellulose column were filtered through an Amicon membrane (10,000 mol wt cutoff) in 20 mM K-phosphate buffer (pH 7.5). Yields were determined spectrophotometrically (Buchanan and Arnon, 1971).

Each experiment utilized 250 mL of reaction buffer containing either 10 μM Fd or 100 μM methylviologen; in addition, superoxide dismutase (15 μg/mL), catalase (15 μg/mL), and NH_4Cl (10 mM) were also present. A stock solution of DPC (100 mM in ethanol) was injected to a final concentration of 4 mM. This brought the reaction buffer to 4% (v/v) ethanol, forcing some O_2 out of solution. Bubbles generated were

removed before injecting the Tris-washed thylakoids and initiating experiments with light. Occasional further injections of degassed thylakoids were made to maintain rates of uptake.

Microcosm Experiments

Mesophyll cells, isolated intact from *Asparagus sprengeri* Regel cladophylls (Colman et al., 1979), were placed in a closed system and given enough $NaHCO_3$ to allow O_2 production to near air saturation levels (50 mM Hepes buffer, pH 7.2). O_2 concentrations then remained fairly constant for up to 8 h. Because O_2 was continuously recycled in this system, we refer to it as a "microcosm." Samples were taken about once every 2 h. At the end of each experiment, additional $NaHCO_3$ was injected to verify photosynthetic competence. This was followed by a brief dark period to examine the rate of dark respiration.

To verify that O_2 uptake and evolution occurred simultaneously, we made use of isotope enrichment studies with ^{18}O-^{18}O, which permitted resolution of these fluxes. Light-stimulated O_2 uptake and production by *Asparagus* mesophyll cells was studied using a VG Gas Analysis (Middlewich, England) MM 14–80 SC magnetic sector mass spectrometer equipped with an aqueous inlet system as described by Miller et al. (1988). The MS was set up to measure ion currents on each of masses 32 ($^{16}O^{16}O$), 36 ($^{18}O^{18}O$), 40 (^{40}Ar), 44 ($^{12}C^{16}O^{16}O$), and 45 ($^{13}C^{16}O^{16}O$), twice per minute. Cells were suspended in 6 mL of 10 mM K-phosphate buffer (pH 7) within a stirred, temperature-jacketed glass cuvette. Labeled O_2 was introduced through a capillary opening by injecting and then removing a small bubble of 98% $^{18}O_2$ (Merck, Sharpe and Dohme, Pointe Claire/Dorval, Canada). Carbonic anhydrase was present at 35 μg/mL, which permitted calculation of the DIC concentration from the measurement of CO_2 alone. O_2 exchange rates were calculated using the equations of Peltier and Thibault (1985).

Other Methods

For experiments needing light, irradiance was about 250 $\mu E\ m^{-2}\ s^{-1}$. Temperature was 25°C except where noted. Chl was determined in 80% (v/v) acetone (Mackinney, 1941) or in *N,N*-dimethylformamide (preferred for *Asparagus* mesophyll cells) (Inskeep and Bloom, 1985).

Water O isotopes were determined after direct conversion to CO_2 by the guanidine hydrochloride method (Dugan et al., 1985). We have extensively tested this procedure with isotope standard water samples, V-SMOW and V-SLAP, and are satisfied that the results obtained are precise and free of any detectable systematic error. Isotopic composition of selected samples was confirmed by CO_2 equilibration in independent analyses performed at the U.S. Geological Survey, Reston, VA.

Calculations and Statistics

O_2 produced in photosynthesis was compared with the source water by one-sample *t* test. For uptake reactions, per mil discrimination factors (*D*) describe the instantaneous difference in isotope ratio between substrate (R_s) and product (R_p):

$$D = (1 - R_p/R_s) \times 1000 \quad (1)$$

D was calculated from the "Rayleigh" equation (after Kroopnick and Craig, 1976):

$$D = \frac{\ln R/R_o}{-\ln f} \times 1000, \quad (2)$$

where *R* is the isotope ratio of the substrate at the time of sampling, R_o is the initial isotope ratio, and *f* is the fraction of substrate unconsumed. When R/R_o is plotted against *f*, a curved line is obtained showing how *R* changes as the substrate is consumed (e.g. as in Fig. 1A for RuBP carboxylation catalyzed by spinach Rubisco). The curvature of the line is proportional to *D*, which is easily obtained as the slope of a regression of $\ln R/R_o \times 1000$ against $-\ln f$ to yield a straight line through the origin (e.g. Fig. 1B). Data from replicate experiments can be pooled for this purpose. In general, comparisons of *R* with R_o where *f* was >0.85 or <0.1 were excluded from the analysis. Note that in any given experi-

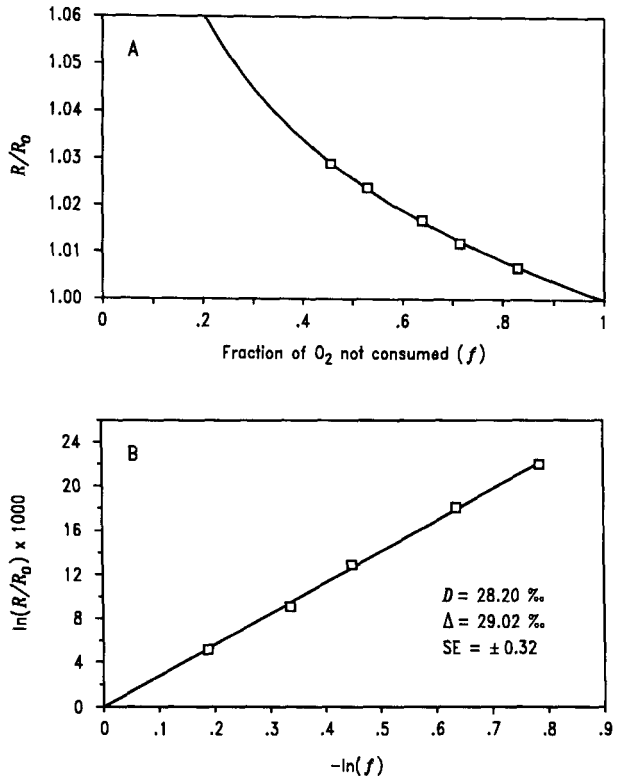

Figure 1. A, Relative change in the isotopic composition of dissolved CO_2 (expressed as R/R_o) as a function of the fraction not consumed (*f*) during carboxylation of RuBP as catalyzed by spinach Rubisco at pH 7.6, 5 mM Mg^{2+}. These are raw data not yet corrected for the HCO_3:CO_2 equilibrium isotope effect. B, The same data plotted in linear form after correcting for the HCO_3:CO_2 equilibrium isotope effect. The fitted regression line is described by $\ln R/R_o = D(-\ln f)/1000$, where the discrimination factor is given by the slope, *D*, which is also presented in "Δ" notation. SE is adjusted as described in Weger et al. (1990).

mental run, R_o need not necessarily be the first sample taken, and any sample measurement may be compared to any preceding measurement. This approach eliminates bias associated with the first sample and gives the best estimate for D. However, because each comparison is not truly independent, degrees of freedom were reduced as described by Weger et al. (1990) to calculate SE and conduct t tests.

For carbon isotope fractionations by Rubisco, a correction for the equilibrium fractionation between HCO_3^- and dissolved CO_2 (Mook et al., 1974) was applied before regression by adjusting R/R_o as follows:

$$R/R_o \text{ adjusted} = \frac{(fR/R_o)^C}{f}, \quad (3)$$

where $C = (1.009 + 10^{(pK-pH)})/(1 + 10^{(pK-pH)})$. This calculation is mathematically equivalent to applying the correction after regression (see "Appendix"). The equation is used here to facilitate graphical comparison of Rubisco oxygenase and carboxylase data on the same scale, but it also permits combining into a single regression analysis data obtained at different pH or different pK. For any given experiment, the pK for the dissociation constant of carbonic acid was adjusted for temperature and ionic strength (Yokota and Kitaoka, 1985).

The use of D as set out in Equation 1 is convenient for purposes of our regression analysis. However, it is now common practice in the botanical literature to express carbon isotope discrimination in "Δ" notation (Farquhar et al., 1989), whereby, on a per mil scale:

$$\Delta = (R_s/R_p - 1) \times 1000 \quad (4)$$

Note that R_s/R_p is equal to the "isotope effect," the ratio of rate constants for reactions of the isotopic substances (i.e. k^{16}/k^{18} or k^{12}/k^{13}). Conversion of D to Δ is straightforward:

$$\Delta = \frac{D}{1 - (D/1000)} \quad (5)$$

To conform to this standard, we here depart from our previous papers and report all final discrimination values in terms of Δ.

RESULTS

O_2 Evolution

The isotopic composition of O_2 evolved in photosynthesis from three types of plant material is presented in Table I. Illuminated spinach thylakoids supplied with $K_3Fe(CN)_6$ as an electron acceptor produced O_2 that was not different from the source water (change in $\delta^{18}O = -0.06 \pm 0.20‰$). In some earlier experiments (not presented), there was no effect of added $NaHCO_3$ and carbonic anhydrase. Tracer studies with $^{18}O_2$-enriched systems indicate that there is no detectable O_2 uptake under these circumstances (J.A. Berry, unpublished data). Therefore, the results demonstrate conclusively that there is no fractionation of O isotopes in the photolysis of water.

The isotopic composition of O_2 continuously sparged from illuminated cultures of *A. nidulans* or *Phaeodactylum tricornutum* Bohlin (a marine diatom) was also not statistically different from source water. The $\delta^{18}O$ of the collected O_2 did, however, tend toward being slightly more positive. The mean differences in $\delta^{18}O$ were $+0.38 \pm 0.27‰$ for *A. nidulans* and $+0.62 \pm 0.23‰$ for *P. tricornutum*. DIC concentrations were kept high in these experiments to minimize photorespiration.

C and O Isotope Discrimination by Rubisco

Isotope discriminations associated with carboxylation and oxygenation of RuBP, as catalyzed by Rubisco in the presence of 20 to 25 mM Mg^{2+}, are compared in Figure 2, A and B. In all cases, substrate concentration was without influence and the presence of the competing substrate was inconsequential. Enzyme from three very dissimilar sources, representative of photosynthetic bacteria (*Rhodospirillum rubrum*), cyanobacteria (*Anacystis nidulans* Richter), and higher plants (spinach), discriminated against ^{18}O by 21.3‰. Though of similar magnitude, O isotope discrimination by Rubisco was statistically different from the Δ for glycolate oxidase ($P < 0.005$).

In contrast to the O isotope results, carbon isotope discrimination by spinach Rubisco was significantly greater ($P \ll 0.001$) than discrimination by *R. rubrum* or *A. nidulans* enzyme (Fig. 2B), which did not differ. Carbon isotope discrimination by spinach Rubisco was 30.3‰ (pH 8.5, 20 mM Mg^{2+}), whereas *R. rubrum* enzyme had a Δ of 23‰ (pH 7.9,

Table I. *Isotopic composition of oxygen evolved in photosynthesis under conditions chosen to minimize concurrent O_2 uptake*

Different plant materials and conditions were used in a series of experiments where, in each case, the $\delta^{18}O$ of the source water (i.e. the suspension buffer), the mean $\delta^{18}O$ of evolved O_2 (with SE and sample size), and the difference between the two are provided.

Plant Material and Conditions	$\delta^{18}O$ of Source Water	$\delta^{18}O$ of Evolved O_2 ±SE (n)	Difference
A. nidulans He-sparged whole cells	16.76‰	17.14 ± 0.27‰ (8)	+0.38‰
P. tricornutum He-sparged whole cells	22.72‰	23.34 ± 0.23‰ (5)	+0.62‰
Spinach thylakoids Closed system, artificial electron acceptor supplied	17.24‰	17.18 ± 0.20‰ (6)	−0.06‰

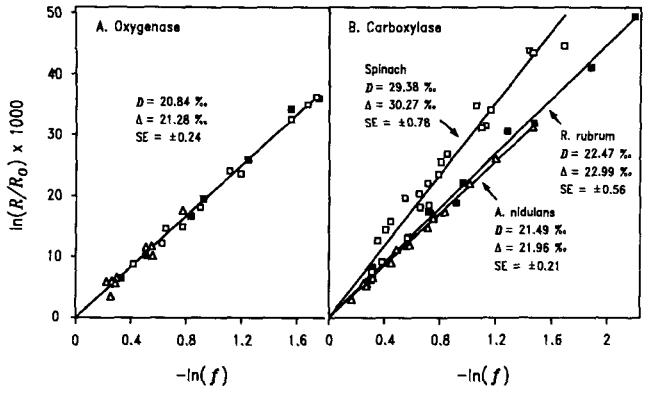

Figure 2. Isotope discrimination by Rubisco from different sources. Discrimination (expressed both as *D* and Δ) and SE values are presented. A, Discrimination against $^{18}O^{16}O$ associated with oxygenase activity. B, Discrimination against dissolved $^{13}CO_2$ associated with carboxylase activity. □, Spinach enzyme (pH 8.5, 20 mM Mg^{2+}); ■, *R. rubrum* enzyme (pH 7.9, 20 [A] and 25 [B] mM Mg^{2+}); Δ, *A. nidulans* enzyme (pH 8.1, 25 mM Mg^{2+}). Carboxylation data were corrected for the HCO_3:CO_2 equilibrium isotope effect prior to plotting.

25 mM Mg^{2+}) and *A. nidulans* enzyme had a Δ of 22‰ (pH 8.1, 25 mM Mg^{2+}). Possible effects of pH and Mg^{2+} concentration were examined in some cases. With spinach enzyme at pH 7.6 and 5 mM $MgCl_2$, Δ was 29.0‰ (Fig. 1) and not statistically different from the value obtained at pH 8.5 and 20 mM $MgCl_2$. However, a reduced $MgCl_2$ concentration (2 mM; pH 7.9) did have a significant effect ($P < 0.001$) on discrimination by the *R. rubrum* enzyme. In this case (data not presented), Δ was 19.59‰. For ease of comparison, Table II presents a summary of all Rubisco discrimination factors determined in this study.

Glycolate Oxidase

O isotope discrimination by spinach glycolate oxidase, with or without added catalase, is shown in Figure 3. Although addition of catalase almost divided the rate of net O_2 uptake in half (not shown), there was no effect on Δ, which was 22.7‰.

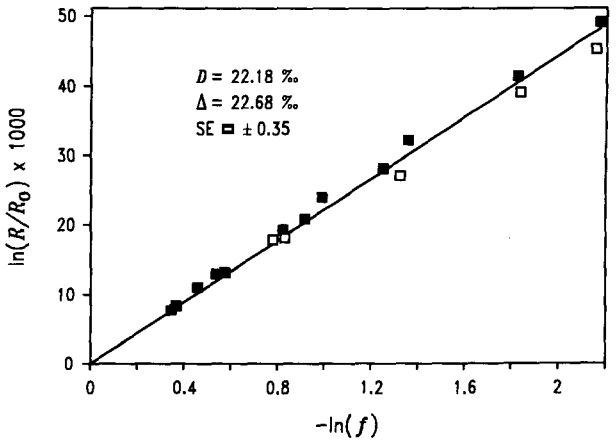

Figure 3. O isotope discrimination by spinach glycolate oxidase in the presence (■) or absence (□) of added catalase. Discrimination (expressed both as *D* and Δ) and SE are shown for the pooled data.

Table II. *Summary of isotope discrimination factors (Δ) for reactions catalyzed by Rubisco as determined in this study*

SE and number of sample:reference comparisons used for each estimate are provided.

Reaction and Enzyme Source	Conditions	Δ	±SE (n)
Oxygenase			
A. nidulans[a]	pH 8.1, 25 mM Mg^{2+}	21.6	±1.2 (9)
R. rubrum[a]	pH 7.9, 20 mM Mg^{2+}	21.4	±0.4 (7)
Spinach	pH 8.5, 20 mM Mg^{2+}	21.1	±0.3 (10)
Overall		21.3	±0.2 (26)
Carboxylase			
A. nidulans[a]	pH 8.1, 25 mM Mg^{2+}	22.0	±0.2 (18)
R. rubrum[a]	pH 7.9, 25 mM Mg^{2+}	23.0	±0.6 (10)
	pH 7.9, 2 mM Mg^{2+}	19.6	±0.4 (24)
Spinach	pH 8.5, 20 mM Mg^{2+}	30.3	±0.8 (22)
	pH 7.6, 5 mM Mg^{2+}	29.0	±0.3 (5)

[a] Expressed in *E. coli*.

Fractionation of O Isotopes in the Mehler Reaction

To measure discrimination during the Mehler reaction, it was necessary to eliminate O_2 evolution. Chloroplast fragments washed with Tris buffer at high strength are not able to split water and produce free O_2 (Yamashita and Butler, 1968). DPC is very effective as an electron donor to PSII in such preparations (Vernon and Shaw, 1969). In our experiments, all capacity for O_2 evolution was eliminated by Tris washing. The production of O_2 was not detectable in the presence of an electron acceptor, uncoupler, and light. Uptake of O_2 in the presence of DPC was fully light dependent. There was some background uptake (also light dependent) in the absence of DPC. This amounted to about 10% of the DPC-sustained rate (with Fd). Exogenous Fd doubled the "ferredoxin-free" rate of O_2 uptake (not shown) when supplied at 10 μM, whereas methylviologen (100 μM) approximately tripled the Fd-free rate of uptake. Higher rates of uptake were not obtainable with higher Fd concentrations.

Discrimination associated with photoreduction of O_2 mediated by either methylviologen or Fd is presented in Figure 4. There was no difference in Δ between these two types of Mehler reactions, and the combined data yield a value of 15.3‰. This Δ is substantially lower than those obtained for glycolate oxidase or the Rubisco oxygenase reaction (significantly different at $P \ll 0.001$).

Microcosm Experiments

When *Asparagus* mesophyll cells provided with CO_2 were allowed to actively photosynthesize within a closed system, the initial difference between the $\delta^{18}O$ of the O_2 produced and the source water was near zero. However, the difference in $\delta^{18}O$ increased rapidly as the O_2/CO_2 ratio decreased and the compensation point was approached (Fig. 5), and continued to rise well after O_2 concentrations had stabilized. Even-

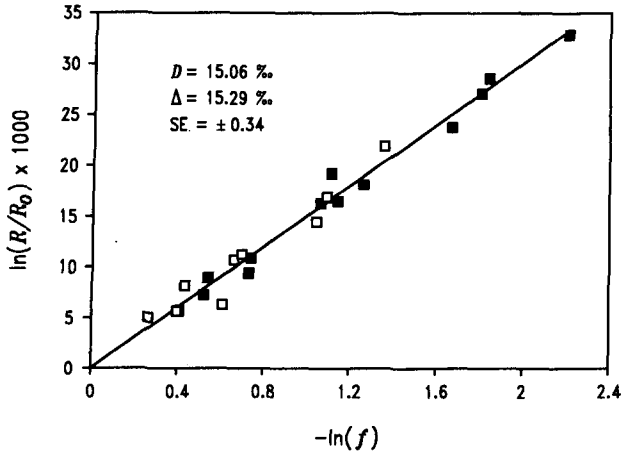

Figure 4. O isotope discrimination by the Mehler reaction as catalyzed by spinach thylakoids in the presence of either 100 μM methylviologen (■) or 10 μM free Fd (□). Discrimination (expressed both as *D* and Δ) and SE are shown for the pooled data.

tually, the isotopic composition also stabilized as ^{18}O accumulated to a level sufficient to counter discrimination, resulting in a plateau $\delta^{18}O$ difference of about +21.5‰. A similar result, however, might have been obtained if the cells had simply lost competence or if gross O_2 exchange had otherwise stopped. This possibility had to be ruled out. A typical O_2 electrode trace from one of the microcosm experiments is presented in Figure 6. The resumption of net O_2 production upon supplying additional bicarbonate (increasing the CO_2/O_2 ratio) demonstrates the continued photosynthetic competence of the cells after prolonged exposure to light at the CO_2 compensation point.

To confirm sustained O_2 turnover at the compensation

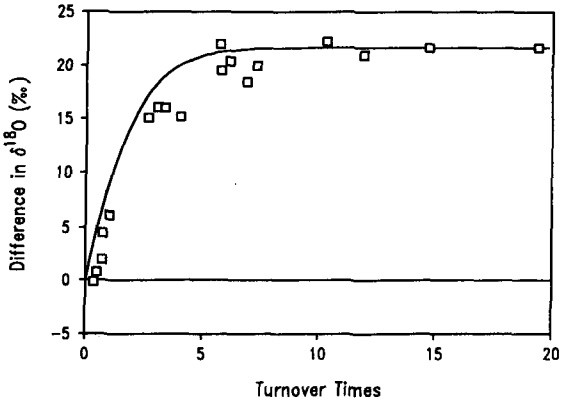

Figure 5. Change in $\delta^{18}O$ of dissolved O_2, relative to source water, in *Asparagus* mesophyll cell microcosm experiments. Data are from six experiments normalized along the *x* axis, where one turnover time is equal to the plateau O_2 concentration divided by the initial rate of O_2 evolution (in practice, 20–60 min). The curved line is the expected change in $\delta^{18}O$ assuming a constant rate of O_2 evolution and a net Δ of 21.7‰ in O_2 uptake. Chl concentration averaged 10.7 μg/mL. Plateau O_2 concentrations ranged from 250 to 640 μM and averaged 360 μM.

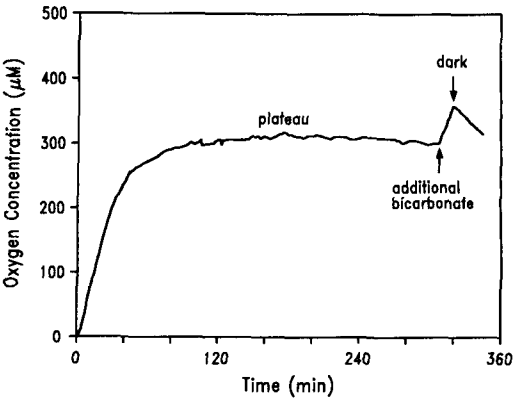

Figure 6. Typical O_2 electrode trace from one of the *Asparagus* mesophyll cell microcosm experiments. Near the end of the initial degassing period, $NaHCO_3$ was injected to 412 μM (a fraction of which was lost to the He stream prior to sealing the reaction vessel at time zero). This supported a rise to a compensation point O_2 concentration of about 310 μM. Sampling for $\delta^{18}O$ was at 32, 178, and 305 min. At 307 min, additional bicarbonate was injected to 750 μM. The ensuing rate of net O_2 evolution equaled the initial rate (57 μmol O_2 mg^{-1} Chl h^{-1}). Illumination ceased at 320 min and the final rate of dark respiration was 8.0 μmol O_2 mg^{-1} Chl h^{-1}. Temperature was 26.5°C. Chl concentration was 8.9 μg/mL.

point, tracer studies with enriched $^{18}O_2$ were conducted to monitor the evolution of $^{16}O_2$ and uptake of $^{18}O_2$. As shown in the top panel of Figure 7, the DIC concentration declined upon illumination, reaching the compensation point within 12 to 15 min. The total O_2 concentration (bottom panel Fig. 7) also reached a plateau (the downward trend beyond this point reflects uptake by the MS inlet). The continued disappearance of $^{18}O_2$ indicates turnover of the total O_2 pool that is replenished as $^{16}O_2$ from photolysis of water. A further injection of $^{18}O_2$ at 74 min uncovered continued $^{16}O_2$ evolution. In darkness, the $^{16}O_2$ and $^{18}O_2$ traces became parallel, as was expected, because $^{16}O_2$ production is light dependent. There was also a decrease in the rate of $^{18}O_2$ uptake, demonstrating that O_2 consumption was stimulated by light (i.e. there was photorespiration). The initial rate of gross O_2 evolution in the experiment shown in Figure 7 was 19.4 μmol O_2 mg^{-1} Chl h^{-1}, decreasing to 11.2 μmol O_2 mg^{-1} Chl h^{-1} at 17.5 min. After 75 min at the compensation point, the gross O_2 exchange rate had decreased to 6.8 μmol O_2 mg^{-1} Chl h^{-1}. The final dark respiration rate was 1.25 μmol O_2 mg^{-1} Chl h^{-1}, which was not much different from an initial rate of 1.17 μmol O_2 mg^{-1} Chl h^{-1} determined before the experiment.

The rate of the Mehler reaction was estimated from changes in $^{18}O_2$ consumption during light to dark transitions at saturating DIC (data not shown). Mean gross O_2 evolution in three experiments was 15.4 μmol O_2 mg^{-1} Chl h^{-1}, and the mean rate of dark respiration was 1.24 μmol O_2 mg^{-1} Chl h^{-1}. Light-stimulated O_2 uptake averaged 3.27 μmol O_2 mg^{-1} Chl h^{-1}, or 21.3% of gross O_2 evolution. Assuming that this proportion is representative of the experiment in Figure 7, we calculate that initially, 68 to 71% of O_2 uptake at the compensation point was by photorespiration, decreasing to 61% by the end of the experiment.

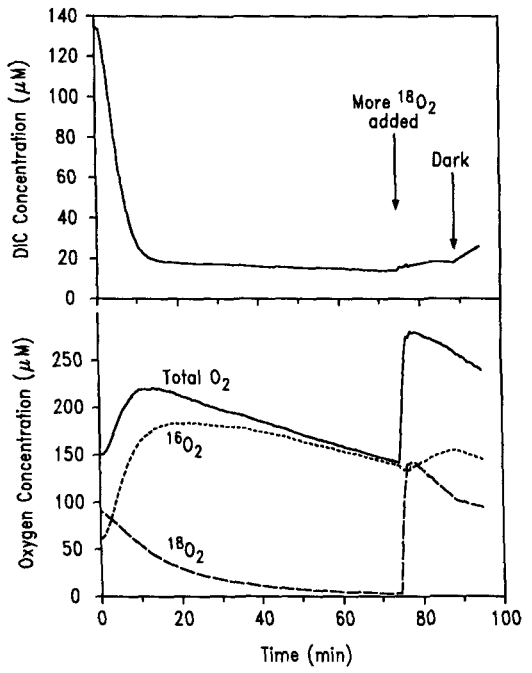

Figure 7. Gas exchange by *Asparagus* mesophyll cells in an $^{18}O_2$ enriched system while approaching and remaining at the compensation point. The upper panel shows changes in the DIC concentration of the buffer medium. The lower panel shows changes in the concentrations of dissolved $^{16}O_2$ (mass 32), $^{18}O_2$ (mass 36), and total O_2. Illumination ceased at 88 min. Chl concentration was 58 μg/mL.

Modeling of Microcosms

To compare the in vitro discrimination data with the results of the in vivo mesophyll cell microcosm experiments, we have modeled expected trends in concentration and isotopic composition of dissolved O_2 in a closed system as it reaches the compensation point (Fig. 8 and solid line in Fig. 5). The model is analogous to one used by Guy et al. (1989b) to calculate discrimination in CO_2 fixation with concurrent CO_2 release in a closed system. O_2 consumption is treated as a negative first-order rate process (with rate constant k_1), and O_2 evolution as a positive zero-order rate process (with rate constant k_2). The O_2 concentration at time t is given by:

$$O_t = (O_o - k_2/k_1)e^{-k_1 t} + k_2/k_1, \quad (6)$$

where O_0 is the initial O_2 concentration. Separate equations of this form can be written for ^{16}O and for ^{18}O, such that k_1^{16} and k_1^{18} are the respective rate constants for photorespiratory O_2 consumption, and k_2^{16} and k_2^{18} are the respective rates of photosynthetic O_2 evolution. The ratio k_2^{18}/k_2^{16} equals the $^{18}O/^{16}O$ ratio of the O_2 produced in photosynthesis (i.e. the isotope ratio of the source water). The ratio k_1^{18}/k_1^{16} is the inverse of the kinetic isotope effect associated with O_2 uptake. By definition, $\Delta = (k^{16}/k^{18} - 1) \times 1000$; hence, after rearrangement, $k_1^{18}/k_1^{16} = 1/(1 + \Delta/1000)$. At the compensation point, rate of uptake must equal rate of evolution, which is assumed to be constant throughout. Given an initial rate of O_2 evolution, the sum of k_1^{18} and k_1^{16} is, therefore, known, and for any given Δ, so is their ratio. Concentrations of $^{18}O^{16}O$, $^{16}O^{16}O$, and total O_2, and $\delta^{18}O$ can then be calculated as a function of elapsed time.

Modeled trends in $\delta^{18}O$ and O_2 concentration using single-step discrimination measurements and appropriate starting conditions (Fig. 8) compare favorably with results for microcosm experiments presented in Figures 5 and 6, respectively. To facilitate comparison, the expected trend in $\delta^{18}O$ assuming constant rates of O_2 evolution and a net Δ of 21.7‰ is superimposed on the data in Figure 5.

DISCUSSION

O_2 Evolution

Results of our experiments with isolated spinach thylakoids definitively show that there is no fractionation of O isotopes in the photolysis of water by PSII. Previous investigators used whole cells or leaves (Dole and Jenks, 1944; Vinogradov et al., 1969; Stevens et al., 1975; Metzner et al., 1979); consequently, there was potential for simultaneous O_2 uptake by the competing processes of respiration, photorespiration, and the Mehler reaction. Results of some of these studies were superficially consistent with a reverse isotope effect favoring the production of isotopically heavy O_2. However, if respiration continues in the light, net O_2 will be enriched (Vinogradov et al., 1960). Because respiration rates are low in photosynthetic tissues, this enrichment is not likely to be more than 2 or 3‰. Vinogradov et al. (1960) made algebraic corrections for respiration and tentatively concluded that the O_2 of photosynthesis was not different from water.

The effect of photorespiration is potentially much more significant. It is interesting to note that Metzner et al. (1979) observed the greatest apparent fractionation of photosyn-

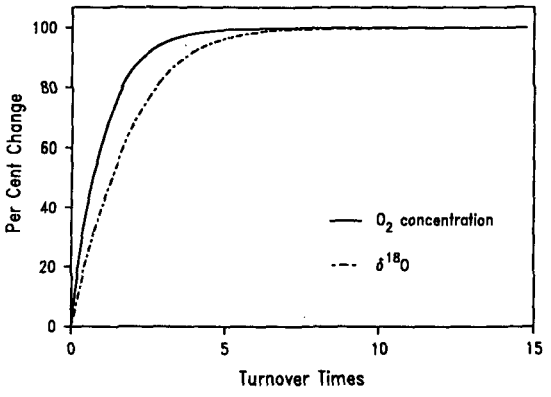

Figure 8. Comparison of expected relative trends in concentration and isotopic composition of dissolved O_2 in a closed system upon approaching and reaching the compensation point. Initial conditions are a near zero O_2 concentration and enough DIC to support O_2 evolution to an eventual equilibrium concentration close to air saturation. Changes in $\delta^{18}O$ are expressed in terms of percent approach to the equilibrium value (i.e. the isotopic compensation point), given no discrimination in O_2 production and a constant positive discrimination during O_2 consumption. One turnover time equals the compensation point O_2 concentration divided by the rate of O_2 evolution, which is assumed to be constant throughout.

thetic O_2 (+15‰) under aerobic conditions known to favor photorespiration (Canvin et al., 1980). Stevens et al. (1975) examined photosynthetic O_2 accumulated by cultures of a cyanobacterium and a green alga at DIC concentrations high enough to keep the rate of photorespiration negligible. The difference in $\delta^{18}O$ between the source water and the collected O_2 was near zero, indicating that there could not be a reverse isotope effect in the photolysis of water. However, because of uncertainty regarding the amount of respiration taking place in the light, the prospect of a small normal isotope effect (favoring ^{16}O production) remained. Note that O_2 collected during He sparging of *A. nidulans* and *P. tricornutum* cultures was slightly enriched (Table I), and the possibility of some residual O_2 uptake under even these conditions cannot be ruled out. The absence of a significant isotope effect, however, suggests that uptake by the algae was minimal. Because *Asparagus* mesophyll cells clotted together when bubbled, it was not possible to use He sparging to examine the possibility of discrimination during in vivo O_2 production by these cells. However, the $\delta^{18}O$ of initial O_2 evolved in the microcosm experiments was very close to the source water, which is consistent with the conclusion that there is no discrimination associated with O_2 evolution per se.

Uptake Reactions Catalyzed by Rubisco

The most important O_2 uptake reaction associated with photosynthesis is oxygenation of RuBP at the active site of Rubisco. This reaction is the initial step in photorespiration and discriminates against ^{18}O by 21.3‰ (Fig. 2). The source of the enzyme, representing a very broad selection of organisms (higher plants, cyanobacteria, and photosynthetic anaerobic bacteria), had no bearing on the results. Using spinach enzyme, Kreckl et al. (1989) examined Rubisco-mediated O isotope fractionation, but obtained markedly higher estimates of Δ, ranging from 28 to 36‰ depending on pH and metal ion. Many more studies of carbon isotope fractionation during CO_2 fixation by this enzyme have been reported (Roeske and O'Leary, 1984, and references therein). The Δ of 30.3‰ we obtained for spinach carboxylase by substrate analysis is in good agreement with the currently accepted best value of 29‰ determined by Roeske and O'Leary (1984), who used product analysis (note that both values are with respect to dissolved, not gaseous, CO_2). This increases our confidence in our determination of the O isotope effect.

Fractionation of carbon isotopes by Rubisco varies with enzyme source, whereas fractionation of O isotopes does not (Fig. 2 and Table II). Compared to spinach Rubisco, discrimination was reduced by about 7‰ when RuBP carboxylation was catalyzed by enzyme from either *R. rubrum* or *A. nidulans*. Roeske and O'Leary (1984, 1985) found a similar but somewhat larger difference between the spinach and *R. rubrum* enzymes. For the latter, these authors report a value of 17.8‰ at pH 7.8 and 10 mM Mg^{2+}.

The different types of Rubisco have different structural and kinetic properties (Pierce, 1989). The higher plant and cyanobacterial enzymes have eight large and eight small subunits, whereas *R. rubrum* enzyme has two large subunits and no small subunits. The enzymes have different relative specificities for CO_2 and O_2. The specificity coefficient of Rubisco (equivalent to the rate of CO_2 fixation divided by the rate of O_2 fixation when these substrates are at equal concentration) varies from 10 to 15 in photosynthetic anaerobes to about 50 in cyanobacteria and 80 in higher plants (Pierce, 1989). This trend reflects differences in the Michaelis constant for CO_2, and it would seem that the substrate specificity of Rubisco has evolved over time to deal with changes in its operational environment. The large kinetic isotope effects associated with both CO_2 fixation and O_2 fixation are consistent with current models of the Rubisco reaction mechanism whereby binding of RuBP occurs to the active site, followed by enolization, and reaction of CO_2 or O_2 with the enolate (Pierce, 1989). Discrimination is presumably at this last step, which is rate limiting. There is potential for limited reversibility at binding of CO_2 to the enolate, and this is most prominent with *R. rubrum* enzyme, consistent with its lower isotope effect (cf. Roeske and O'Leary, 1985). We speculate that Rubisco has evolved to "speed" the slow step for carboxylation and not oxygenation. This would move some control to other steps in the carboxylation reaction, resulting in differences in Δ for carbon but not for O.

Data of Winkler et al. (1982) and Roeske and O'Leary (1984) indicate that Mg^{2+} concentration and pH may influence the Rubisco carbon isotope effect. Our studies indicate that the influence of pH, at least, is small and not likely to be important in vivo. The effect of Mg^{2+} concentration is more uncertain. We did not detect a significant effect of a combination of lower pH (7.6 instead of 8.5) and reduced Mg^{2+} concentration (5 mM instead of 20 mM) on discrimination by spinach Rubisco. However, a reduction in Mg^{2+} concentration from 20 to 2 mM (both at pH 7.9) reduced Δ from 23 to 19.6‰ for *R. rubrum* Rubisco (Table II).

Glycolate Oxidase

O_2 consumption in photorespiration of phosphoglycolate produced by Rubisco oxygenase also contributes to isotope discrimination by plants. Glycolate oxidase, with or without added catalase, discriminated by 22.7‰ (Fig. 3). The products of this reaction are glyoxylic acid and hydrogen peroxide. In vivo, catalase promotes the decomposition of the H_2O_2 to yield H_2O and ½ O_2. The net effect is that for every two O_2 taken up by Rubisco, one additional O_2 is consumed by glycolate oxidase. The lack of any isotope fractionation by added catalase confirms Dole's (1952) observations on enzymic decomposition of H_2O_2.

It is also possible that there will be an increase in dark respiration resulting from glycine decarboxylation in the mitochondria. This could result in at most a further 0.5 O_2 being consumed for every two O_2 fixed by Rubisco. However, in photorespiration, NADH produced during the decarboxylation of glycine in the mitochondria is balanced by the consumption of NADH during reduction of hydroxypyruvate in the peroxisome, and these two reactions may be linked by a dicarboxylate shuttle (Badger, 1985), with little or no additional O_2 uptake by the mitochondrial electron transport chain. Discrimination by dark respiring *Asparagus* mesophyll cells was 20.6‰ (Guy et al., 1989a). Assuming the above stoichiometry, the expected overall Δ for photorespiration is

between 21.6 and 21.7‰, the latter corresponding to no net increase in dark respiration.

Mehler Reaction

In chloroplasts, some photosynthetic energy is dissipated in an autooxidation by molecular O_2 of the reduced components of PSI, principally Fd (Badger, 1985). This process is referred to as the Mehler reaction. An artificial Mehler reaction may also be supported by autooxidation of methylviologen, which accepts electrons specifically from the reducing end of PSI. The initial product of the Mehler reaction is superoxide (O_2^-), which is rapidly consumed in vivo by superoxide dismutase. This enzyme catalyzes the conversion of two O_2^- molecules to one O_2 and one H_2O_2. Chloroplasts lack catalase, so the H_2O_2 produced is detoxified by a series of reactions beginning with an ascorbate peroxidase (Badger, 1985). Unlike H_2O_2 peroxidation via catalase, these reactions yield no O_2. To mimic this system and achieve the correct stoichiometry using spinach thylakoids, we provided superoxide dismutase, catalase, and 4% ethanol. With ethanol present, catalase yields acetaldehyde (rather than O_2 and H_2O) from H_2O_2 (Trebst, 1972). Isotope discrimination in the Mehler reaction, whether mediated by Fd or by methylviologen, was about 15.3‰ (Fig. 4). Though substantial, this value is lower than for any other major O_2 uptake reaction in plants.

O Isotope Fractionation at the Compensation Point

When *Asparagus* mesophyll cells approached the compensation point within a sealed vessel, the $\delta^{18}O$ of dissolved O_2 diverged rapidly from the source water (Fig. 5). In such a closed system, the ^{18}O concentration will slowly build up until its increased abundance exactly counters the discrimination against it. For $\Delta = 21.7‰$ (the weighted mean for photorespiration), the plateau $\delta^{18}O$ at which this will occur is at an ^{18}O abundance 1.0217 times that of freshly evolved O_2. That is, the $\delta^{18}O$ of the dissolved O_2 pool will approach a value 21.7‰ heavier than that of the original source. The O_2-water difference in $\delta^{18}O$ did indeed level off close to this expected value under conditions where the continued compensation point turnover of O_2 could be demonstrated (Figs. 6 and 7).

Model results (Fig. 8) indicate that it should have taken 4.7 turnover times to reach the plateau $\delta^{18}O$ to within 1‰, and 7.5 turnover times to within 0.1‰. The change in $\delta^{18}O$ lagged behind the modeled trend (solid line in Fig. 5) most probably because gross O_2 evolution was not constant and declined somewhat at the compensation point, as occurred during the tracer studies (Fig. 7). The model would also overestimate initial rates of photorespiration where O_2 concentrations are low and CO_2 concentrations are high. The model was constructed primarily to follow the approach to final isotopic steady state, which occurred at constant CO_2 concentration.

The expected trend shown in Figure 5 assumes a constant Δ of 21.7‰ and considers photorespiration only. Although photorespiration is certainly the most important O_2 uptake process at the compensation point, it is not the only one. In leaves or cells from a number of higher plants, 60 to 70% of the compensation point O_2 uptake can be attributed to photorespiration (Canvin et al., 1980; Behrens et al., 1982; Furbank et al., 1982; Carrier et al., 1989). Our results for *Asparagus* mesophyll cells were very comparable. The tracer studies indicated that approximately one-third of the compensation point O_2 uptake was nonphotorespiratory, of which mitochondrial respiration accounted for as much as half, with the balance being ascribed to the Mehler reaction. Given a Δ of 15.3‰ associated with the Mehler reaction (Fig. 4) and a Δ of 20.6‰ for respiration by *Asparagus* mesophyll cells (Guy et al., 1989a), the weighted mean Δ for total compensation point O_2 uptake should actually be 19.8 to 20.3‰. The data in Figure 5 demonstrate that overall fractionation was at least equal to this and support the conclusion that isotope discrimination by photorespiration is fully expressed in vivo. Unlike the other reactions, there is no stoichiometric constraint on the Mehler reaction, and a better fit to the observed data could be obtained by assuming that less than one-sixth of total O_2 uptake occurred by this reaction.

Implications for the Dole Effect and Measurements of Gas Exchange

The plateau difference in $\delta^{18}O$ of about 21.5‰ obtained in the *Asparagus* mesophyll cell microcosm experiments resembles a "Dole effect" within the confines of the reaction vessel. This value is close but not equal to the 23.5‰ difference between the $\delta^{18}O$ of atmospheric O_2 and the $\delta^{18}O$ of ocean water (Kroopnick and Craig, 1972). A complete correspondence is not to be expected for several reasons. First, the isotopic steady state reached within the reaction vessel is predominantly the result of photorespiration, whereas globally, dark respiration plays a greater role. Although a small portion of global respiration is mediated by the alternative oxidase, and this enzyme has a large Δ (24–26‰), the predominant respiratory enzyme is Cyt oxidase, which has a smaller discrimination of 16 to 19‰ (Guy et al., 1989a, and references therein), slightly lowering enzymic effects on global discrimination. Second, the effects of the hydrologic cycle on chloroplast water (^{18}O depletion during precipitation and ^{18}O enrichment during evapotranspiration) must be added to the enzymic effect. It has been estimated that net fractionation in precipitation and evapotranspiration contributes 3 to 4‰ more to the global Dole effect (Förstel, 1978).

Dark respiration has previously been invoked as the major and even the only cause for the enrichment of ^{18}O in atmospheric O_2. However, it is not quite possible to account for the Dole effect based on hydrology and respiration alone. Our work demonstrates that photorespiration is also a major cause of the Dole effect, and that the Mehler reaction should not be ignored. Besides contributing to an improved understanding of the biogeochemical O cycle, results presented here may bear upon several problems in plant physiology. For example, differences in Δ between respiration, photorespiration, and the Mehler reaction may find application in resolving pathways of O_2 uptake during gas exchange, and discrimination associated with photorespiration could be used to examine photorespiratory activity and its in vivo regulation.

APPENDIX

Algebraic Derivation of a Formula to Directly Correct R/R_o for Contributions due to the HCO_3:CO_2 Equilibrium Isotope Effect

Note that the kinetic isotope effect is k^{12}/k^{13}, the ratio of rate constants for reactions involving the respective isotopes. Note also that:

$$k^{12}/k^{13} = R_s/R_p \quad (1)$$

where R_s and R_p are the $^{13}C/^{12}C$ ratios of source and product, respectively. The equilibrium isotope effect, which we designate C, is K^{12}/K^{13}, and:

$$C = K^{12}/K^{13} = R_s/R_p = R_{HCO_3}/R_{CO_2} \quad (2)$$

where HCO_3^- is the "source" and CO_2 is the "product." As defined in "Materials and Methods," the discrimination factor D is:

$$D = \frac{\ln R/R_o}{-\ln f} \times 1000, \quad (3)$$

which is related to the inverse of the isotope effect as follows:

$$D = (1 - [k^{13}/k^{12}]) \times 1000 \quad (4)$$

When an equilibrium fractionation precedes a kinetic fractionation, the overall isotope effect is equal to the product of the two (O'Leary, 1981):

$$(k^{12}/k^{13})_T = (K^{12}/K^{13})_C \times (k^{12}/k^{13})_E \quad (5)$$

where subscripts T, C, and E indicate total, HCO_3:CO_2 equilibrium, and enzyme (Rubisco) isotope effects, respectively. The inverse of Equation 5 must also hold:

$$(k^{13}/k^{12})_T = (K^{13}/K^{12})_C \times (k^{13}/k^{12})_E \quad (6)$$

Noting that $(K^{13}/K^{12})_C = 1/C$, Equation 6 rearranges to:

$$(k^{13}/k^{12})_E = C \times (k^{13}/k^{12})_T \quad (7)$$

From Equations 3 and 4 we obtain:

$$k^{13}/k^{12} = 1 + \frac{\ln R/R_o}{\ln f}, \quad (8)$$

which, rearranged, is $\ln R/R_o = \ln f \times (k^{13}/k^{12} - 1)$, or:

$$\ln R/R_o = (k^{13}/k^{12})\ln f - \ln f \quad (9)$$

If there were no HCO_3:CO_2 equilibrium isotope effect, k^{13}/k^{12} in Equation 12 would be $(k^{13}/k^{12})_E$. As such, changes in R/R_o would be due solely to Rubisco. This adjusted quantity, $(R/R_o)_{adj}$, is what we seek. Equation 9 can be rewritten:

$$\ln (R/R_o)_{adj} = (k^{13}/k^{12})_E \ln f - \ln f \quad (10)$$

Substituting for $(k^{13}/k^{12})_E$ from Equation 7, the above becomes:

$$\ln (R/R_o)_{adj} = (C \times [k^{13}/k^{12}]_T \times \ln f) - \ln f \quad (11)$$

Noting from Equation 8 that $(k^{13}/k^{12})_T = 1 + (\ln R/R_o)/(\ln f)$, we substitute into Equation 11 to obtain:

$$\begin{aligned} \ln (R/R_o)_{adj} &= \left(C \times \left[1 + \frac{\ln R/R_o}{\ln f}\right] \times \ln f\right) - \ln f \\ &= C(\ln f + \ln R/R_o) - \ln f \\ &= (C \ln f) + (C \ln R/R_o) - \ln f \end{aligned} \quad (12)$$

Taking the antilog of both sides of Equation 12 gives:

$$\begin{aligned} (R/R_o)_{adj} &= e^{([C\ln f]+[C\ln R/R_o]-\ln f)} \\ &= (f^C \times [R/R_o]^C)/f \\ &= \frac{(f \times R/R_o)^C}{f} \end{aligned} \quad (13)$$

At any f, Equation 16 can be used to correct R/R_o for the effects of the HCO_3:CO_2 equilibrium. However, the value of C, the equilibrium isotope effect, is influenced by the pH of the medium and by the pK for carbonic acid. Mook et al. (1974) state that at 25°C (there is a strong temperature dependence), fractionation (ε) is:

$$\varepsilon = (R_{CO_2}/R_{HCO_3} - 1) \times 10^3 = -8.97$$

Therefore, $R_{CO_2}/R_{HCO_3} = 0.99103$, and $C = 1.0090511$. This is before correcting for pH and pK. The degree to which the equilibrium fractionation will be expressed depends directly on the proportion of DIC that is HCO_3^-, which is given by:

$$1/(1 + 10^{pK\text{-}pH}) \quad (14)$$

Thus,

$$C = 1 + \frac{0.0090511}{1 + 10^{pK\text{-}pH}} \quad (15)$$

We can put Equation 15 into a different form:

$$C = \frac{1 + 10^{pK\text{-}pH}}{1 + 10^{pK\text{-}pH}} + \frac{0.0090511}{1 + 10^{pK\text{-}pH}} = \frac{1 + 10^{pK\text{-}pH} + 0.0090511}{1 + 10^{pK\text{-}pH}}$$

And, with slight rounding off, we obtain:

$$C = (1.009 + 10^{pK\text{-}pH})/(1 + 10^{pK\text{-}pH}) \quad (16)$$

Equation 16 is the same correction factor employed by Winkler et al. (1982).

ACKNOWLEDGMENTS

We would like to thank S. Freas, T. Hoering, and H. Weger for technical assistance and advice. J. Brown, L. Green, S. Gutteridge, and G. Lorimer kindly donated experimental materials. C. Kendall (U.S. Geological Survey) provided independent verification of water $\delta^{18}O$ values. We thank D. DesMarais (National Aeronautic and Space Administration, Ames Research Center, Moffett Field, CA) for use of isotope ratio mass spectrometer to analyze some samples. Access to the VG Gas Analysis MM 14–80 SC mass spectrometer was provided by the Department of Biology, Queen's University, Kingston, Canada.

Received June 30, 1992; accepted August 24, 1992.

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